Chapter 6

The NER protein Rad33 shows functional homology to human Centrin2 and is involved in modification of Rad4

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The NER protein Rad33 shows functional homology to human Centrin2 and is involved in modification of Rad4.

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Abstract

In the yeast *Saccharomyces cerevisiae* the Rad4-Rad23 complex is implicated in the initial damage recognition of the Nucleotide Excision Repair (NER) pathway. NER removes a variety of lesions via two subpathways: Transcription Coupled Repair (TCR) and Global Genome Repair (GGR). We previously showed that the new NER protein Rad33 is involved in both NER subpathways TCR and GGR. In the present study we show UV induced modification of Rad4 that is strongly increased in cells deleted for *RAD33*. Modification of Rad4 in *rad33* cells does not require the incision reaction but is dependent on the TCR factor Rad26. The predicted structure of Rad33 shows resemblance to the Centrin homologue Cdc31. In human cells, Centrin2 binds to XPC and is involved in NER. We demonstrate that Rad4 binds Rad33 directly and via the same conserved amino acids required for the interaction of XPC with Centrin2. Disruption of the Rad4-Rad33 interaction is sufficient to enhance the modification of Rad4 and results in a repair defect similar to that of a *rad33* mutant. The current study suggests that the role of Rad33 in the Rad4-Rad23 complex might have parallels with the role of Centrin2 in the XPC-HHR23B complex.
6.1 Introduction

Nucleotide Excision Repair (NER) is a DNA repair system characterized by its ability to remove various structurally unrelated lesions from DNA, including the UV induced cyclobutane pyrimidine dimers (CPDs) and pyrimidine pyrimidone (6-4) photoproducts. Completion of the core-NER reaction requires a set of 15 highly conserved proteins. In the yeast Saccharomyces cerevisiae the reaction is initiated by binding of the Rad4-Rad23 complex to the lesion (Guzder et al., 1998; Jansen et al., 1998; Min and Pavletich, 2007; Sugasawa et al., 1998). This event triggers the recruitment of the other NER factors to the site of the damage. Once the DNA adjacent to the lesion is locally unwound, single strand incisions are made on both sides of the damage, followed by the removal of the oligonucleotide containing the lesion. The DNA is restored to its pre-damaged state by the actions of DNA polymerase and ligase (de Laat et al., 1999; Guzder et al., 1995; Park and Choi, 2006; Volker et al., 2001). Defects in the human NER system lead to severe disorders, mostly associated with a highly elevated risk of skin cancer (de Boer and Hoeijmakers, 2000; Kraemer et al., 2007; Leibeling et al., 2006).

Lesion removal by NER is not homogenous as relative fast repair by the subpathway Transcription Coupled Repair (TCR) occurs only in DNA which is transcriptionally active. Global Genome Repair (GGR) on the other hand is slower and removes lesions throughout the entire genome. The TCR pathway, in yeast mainly dependent on the CSB homologue Rad26 (van Gool et al., 1994), is initiated when the RNA polymerase is obstructed at the site of the damage. The presence of this highly lethal structure triggers the recruitment of NER proteins to the site of the lesion (Sarasin and Stary, 2007; Svejstrup, 2002). The second NER subpathway, GGR, requires the Rad7-Rad16 complex in yeast (Verhage et al., 1996) and is suggested to function as a damage sensor in the context of chromatin (Guzder et al., 1997). In humans, no homologues of Rad7 and Rad16 are identified, but DDB1 and DDB2 appear to be the functional equivalents of the yeast GGR proteins (Gillette et al., 2006). Yeast cells lacking the Rad7-Rad16 complex or the Rad26 protein can still rely on TCR or GGR respectively and are therefore only partially UV sensitive. Mutations in genes encoding for core NER factors on the other hand lead to a complete NER defect. In recent years various studies challenged the traditional view of the GGR and TCR systems. For instance, the Rad7 and Rad16 proteins have been shown to function in a post-incision event (Yu et al., 2004) and the TCR factor Rad26 can contribute to repair of the non-transcribed strand in transcriptionally inactive genes (Li et al., 2007).

Although NER is similar in yeast and humans, the role of Rad4 differs from that of its homologue XPC. In yeast TCR is dependent on the Rad4 protein whereas in human cells repair of the transcribed strand can be completed without XPC. Rad4 is therefore considered a core NER protein and XPC a GGR factor. The basis for the different requirement of Rad4 or XPC in transcription coupled repair remains to be elucidated. In yeast, the binding of the Rad4-Rad23 complex to damaged DNA occurs through Rad4 (Guzder et al., 1998; Jansen et al., 1998), a process recently visualized by elegant work showing the crystal-structure of Rad4 bound to DNA containing a CPD lesion (Min and Pavletich, 2007). Rad23 provides stability to Rad4 and enhances its affinity for DNA (Xie et al., 2004). An additional role of Rad23 in NER is mediated via the ubiqui-
uitin-like domain (UbL domain) and two ubiquitin associated domains (UBA domains), which link NER to the ubiquitin/proteasome pathway (Reed and Gillette, 2007; Schauber et al., 1998).

Although a defined role for the ubiquitin proteasome system in NER is not yet clear (Bergink et al., 2007), it was shown that ubiquitylation is involved in damage recognition in human NER. The GGR factor UV-DDB consists of the DDB1 and DDB2 proteins which can bind Cul4A and Roc1 to form a Cullin-RING ubiquitin ligase (Petroski and Deshaies, 2005; Shiyanov et al., 1999). After binding of UV-DDB to a lesion XPC is recruited and ubiquitylated by the DDB1-DDB2-Cul4A-Roc1 complex. At the same time the UV-DDB-ubiquitin ligase complex is auto-ubiquitylated, leading to the degradation of DDB2. The modified XPC is stable and possesses increased affinity for the damage, subsequently replacing UV-DDB at the site of the lesion (Sugasawa, 2006; Wang et al., 2005).

There are indications that ubiquitylation is also involved in GGR of *S. cerevisiae*. The Rad7-Rad16 complex is thought to be part of a Cullin-RING ubiquitin ligase (Ho et al., 2002; Ramsey et al., 2004) similar to the UV-DDB complex. The Rad7-Rad16 ubiquitin ligase was purified and shown to be able to mono-ubiquitylate Rad4 in vitro (Gillette et al., 2006). Whether ubiquitylation plays a role in Rad4 damage recognition is not clear however, since mutation of the SOCS site in Rad7, essential for its function in the Cullin-RING ligase, does not lead to increased UV sensitivity (Gillette et al., 2006).

We previously reported that Rad33, a 20kD protein with no clear homology to any known repair factor, is involved in NER in *S. cerevisiae* (den Dulk et al., 2006). Deletion of *RAD33* leads to moderate UV sensitivity, however, a synergistic effect is observed in the rad23rad33 double mutant which displays UV sensitivity indicative of a complete NER defect. In *rad33* cells only partial repair of the transcribed strand (TS) and no repair of the non-transcribed strand (NTS) is detected implicating that Rad33 is involved in both GGR and TCR (den Dulk et al., 2006).

Large scale affinity capture studies show co-purification of Rad33 with both Rad4 and Rad23 (Krogan et al., 2006) indicating that Rad33 might be part of the Rad4-Rad23 complex. Our previous results show that deletion of *RAD33* causes a reduction of Rad4 protein levels (den Dulk et al., 2006). Here we study the role of Rad33 in relation to the Rad4 protein in more detail. We show that the Rad4 levels in wildtype cells as well as the reduced levels of Rad4 in a *rad33* background are reasonably stable. Furthermore, we find that deletion of *RAD33* strongly enhances UV induced modification of Rad4 in vivo. Interestingly, the data in this paper suggests that Rad33 and the human XPC binding protein Centrin2 might have a comparable role in the NER damage recognition complex.
6.2 Materials and methods

6.2.1 Strains and plasmids

The strains used in this study are listed in table 1. The RAD4TAP rad33 mutants with an additional deletion of either RAD6, UBC4, UBC5, UBC7, UBC8, UBC11, UBC12, or UBC13 were made by crossing MGSC825 cells with BY4741 cells that were deleted for one of the above mentioned genes (Euroscarf). Spores were dissected to obtain haploid cells. YEp112K was constructed by insertion of the EcoRV-PvuII KANMX fragment from pUG6 (Guldener et al., 1996) into the BstXI site of YEp112 (Hochstrasser et al., 1991). TAP tag constructs were created as described previously (Puig et al., 2001). Cells expressing the amino-terminally HA tagged RAD4 gene were created by targeting RAD4 with a modified version of the pBS1761 plasmid (Puig et al., 2001) in which the TAP tag sequence was replaced by a triple HA-tag. The rad4AAA mutations were introduced via the two-step gene replacement method (Sherman, 2002) using the YIpLAC211 vector containing the RAD4 sequence encoding the carboxy-terminal part of Rad4 in which the residues W649, L652 and L656 were replaced by alanines.

6.2.2 Two hybrid experiments

For protein interaction studies the Clontech Matchmaker 3 system was used. The full length RAD23 and RAD33 genes were fused to the GAL4 activating domain (AD) in pGADT7. The RAD4 region coding for amino acids 1-277, the conserved part of RAD4 coding for residues 274-667 and the full length RAD23 gene were fused to the GAL4 binding domain (BD) in pGBK7. pGBK7-RAD4AAA was created as described for the conserved RAD4 region (coding for residues 274-667).

The AD and BD vectors and constructs were introduced into Y187 and AH109 respectively by LiAc transformation. Mating was used to create diploid cells with combinations of AD and BD plasmids. Cultures of diploids were spotted on plates selecting for the presence of two plasmids and on plates indicating expression of the reporter genes (GAL1UAS-GAL1TATA-HIS3; GAL2UAS-GAL2TATA-ADE2). Plates were incubated for 3 days at 30°C.

6.2.3 Western blot analysis

All cell cultures were grown for three days in YPD before extracts were prepared. Optical densities were typically around an OD_{600} of 10 and were found comparable for all strains used. Cells were pelleted and proteins were extracted using 20% TCA as described previously (den Dulk et al., 2006; Falconi et al., 1993). The protein extracts were run on 7.5% SDS page gels and transferred to a PVDF transfer membrane (GE healthcare Hybond-P) using a semi-dry western blotting set (Sigma-Aldrich). The presence of TAP-tagged proteins was detected using rabbit Peroxidase-anti-Peroxidase antibodies (American Qualex, P2250). The blots were stripped and re-probed with an antibody against alpha tubulin as loading control (Genetex, GTX76511). Native Rad4 was shown using anti-Rad4 antibody (Gillette et al., 2006), a kind gift from Dr. Simon Reed. For these blots an unknown protein which reacted aspecifically with anti-Rad4 was used as loading control.

Mouse anti-Ubiquitin antibodies used were purchased from Zymed laboratories (Catalog No. 13-1600), Goat anti-ubiquitin was purchased from Abcam (ab14372).
For the western blot detection of ubiquitin the blots were chemically denatured prior to detection to enhance the sensitivity as described previously (Pagano, 1997). All western blots were developed using Pierce supersignal west Pico and exposed to hyperfilm (GE healthcare) for 30 minutes or overnight for detection of Rad4 modification in wild-type background. For quantitative analysis used in the results sections 3.1 and 3.2 western blots were analyzed with a BioRad Chemidoc XRS and quantified using BioRad Quantity one.

Table 1: *S. cerevisiae* strains used:

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^aRemainder of the genotype identical to that of W1588-4a
^bConstructed as described previously (den Dulk et al., 2006)
^cRemainder of the genotype identical to that of BY4742
3 Results

3.1 Levels and stability of Rad4 in rad33 background.

The steady state protein levels of Rad4 are lower in the absence of Rad33 (den Dulk et al., 2006) (Fig. 1A). The most obvious explanation for this effect is that Rad4 is unstable in rad33 cells. We here examined the levels of Rad4 in wildtype and rad33 cells incubated in the presence of cycloheximide (CHX), blocking de novo protein synthesis.

Figures 1B and 1C show the Rad4 protein levels in wildtype and rad33 cells respectively. Quantifications of the blots are shown in the right panels (Fig 1BC). Figure 1D shows data similar to those in figures 1B and 1C but here extracts from wildtype and rad33 cells are loaded on the same gel (only with CHX) in order to be able to compare the levels in the different backgrounds. Consistent with our previous results (den Dulk et al., 2006) and the data in figure 1A, figure 1D shows that the amount of Rad4 is about 3 fold lower in Rad33 deficient cells. Furthermore, the quantification data (Fig. 1B-D, right panels) indicate that CHX treatment does not markedly influence the levels of Rad4 protein in wildtype or rad33 cells. We find similar results when the native Rad4 levels in wildtype and rad33 cells are compared using anti-Rad4 antibody. The α-specific band which is visible using the Rad4 antibody functions as loading control.

3.2 Rad4 is modified after UV irradiation in rad33 cells.

The stability of Rad4 in rad33 cells discussed above (Fig. 1B-E) is not altered after UV irradiation (Fig. 2A). Interestingly, longer exposures of the blot shown in figure 2A reveal a clear UV induced modification of Rad4 in rad33 cells (Fig. 2B). The modification of Rad4 is visible at low doses of UV, starting at 2 J/m² and does not further increase with doses higher than 8 J/m² (Fig. 2D). Under the same conditions, no modification of Rad4 is observed in wildtype cells (Fig. 2C) or in rad23 cells (data not shown). In rad33 cells exposed to a UV dose of 8 J/m² the modification appears ~5 minutes after UV irradiation and reaches a maximum around 25 minutes after UV treatment (Fig. 2F). During identical treatment Rad4 modification is not detected in Rad33 proficient cells. However, prolonged exposure of the film also reveals some UV
irradiation induced modification of Rad4 in wildtype background (Fig. 2G).

Based on quantification of several western blots we estimate that in rad33 cells the ratio of modified Rad4 in relation to non-modified Rad4 is increased ~50 fold compared to the ratio in wildtype cells. In the absence of Rad33 roughly 10% of the Rad4 protein is modified 30 minutes after UV irradiation. After 120 minutes, the Rad4 modification in rad33 cells was reduced to ~4%. In wildtype cells ~3 fold more Rad4 protein is present, here we find a modified fraction of only ~0,2%. Thus, the absolute amount of modified Rad4 in wildtype cells is ~15 fold lower compared to the amount...
Figure 2
Western blot analysis. TCA extracts of cells with TAP-tagged Rad4 were analysed on western blot using PAP antibodies. Cells were suspended in Phosphate Buffered Saline (PBS), irradiated and resuspended in YPD. At time-points indicated, cells were harvested and TCA extracts were prepared.

(A) Rad4TAP levels in rad33 cells analysed 0, 30, 60 and 120 minutes after UV irradiation or mock treatment. Cells were recovered in the presence of cycloheximide.

(B) as (A), but showing a longer exposure of the film.

(C) Rad4TAP in wildtype cells after UV irradiation with the doses indicated. Cells were irradiated in PBS and were recovered in YPD for 30 minutes.

(D) As (C), but for rad33 cells.

(E) Rad4TAP in wildtype cells irradiated with 8 J/m² harvested at the indicated time points after irradiation.

(F) as (E), but for rad33 cells.

(G) Left panels: Rad4TAP from rad33 cells 0, 30, 60 and 120 minutes after UV irradiation. The lower left panel is identical to the upper panel with the exception of the exposure time. The right panels are similar to the left panels but here Rad4TAP from wildtype cells is analysed. Quantification indicates that, relative to the band intensity of the unmodified Rad4, the modification is ~50 times more intense in rad33 cells compared to that in wildtype cells.

(H) Rad14TAP in UV irradiated (+) or mock treated (-) wildtype or rad33 cells. Cells were recovered in YPD for 30 minutes before preparation of TCA extracts.

(I) As (H), but for TAP-Rad23.
in *rad33* cells.

Two other TAP tagged NER proteins involved in the early steps of NER, Rad14 and Rad23, are not modified in irradiated *rad33* cells, indicating that the UV induced modification, which is strongly increased in *rad33* cells, is not a general effect (Fig. 2H and I).

### 3.3 The nature of the Rad4 modification

It was reported that XPC, the human homologue of Rad4, is ubiquitylated upon UV irradiation (Sugasawa *et al.*, 2005; Sugasawa, 2006; Wang *et al.*, 2005) and ubiquitylation of Rad4 was demonstrated *in vitro* (Gillette *et al.*, 2006). It is therefore possible that ubiquitin is also involved in the modification of Rad4 we observe here. To examine this, modified Hemagglutinin (HA) tagged Rad4 was purified from wildtype and *rad33* cells (Fig. 3A) and analyzed on western blot for interaction with anti-ubiquitin antibodies. However, no signal could be detected when the blot was probed with anti-ubiquitin antibodies from two different suppliers (data not shown). In an alternative approach beads coated with UBA (Ubiquitin Associating) domains were used, which were shown to bind to ubiquitin or ubiquitylated proteins (Wilkinson *et al.*, 2001). Cell extracts of UV irradiated *rad33* cells were incubated with the UBA coated beads. The bound fraction of the beads incubated with extracts from UV irradiated cells shows a marked increase of the modified species of Rad4 whereas these bands are hardly detectable in the bead-bound fraction of cell extracts from unirradiated *rad33* cells. From quantification of the blots it is estimated that for the UV irradiated cells the ratio of modified versus non-modified Rad4 is increased ~8 fold in the bead bound fraction compared to the whole cell extract. The binding to the UBA beads was most clear using extracts from cells in which ubiquitin is overexpressed (Fig. 3B). To check whether the UBA beads actually enriched the ubiquitylated protein pool we stripped the

![Figure 3](image)

**Figure 3**

(A) HA-Rad4 immunoprecipitation. Wildtype and *rad33* cells expressing a genomic amino-terminally HA tagged RAD4 gene were grown for three days. Cell extracts were made in RIPA buffer using glass-beads. Cell extracts were bound for three hours to ProtA beads (Amersham) coated with 12CA5 anti-HA antibodies. The beads were washed and the remaining proteins were boiled in loading buffer, run on 7.5% SDS/PAGE and analysed on western blot. Western blots were probed with 12CA5 anti-HA antibodies.

(B) UBA-beads binding experiment. Cell extracts from UV irradiated or mock treated Rad4TAP*rad33* cells in which additional ubiquitin is expressed from a YEp112K plasmid (Hochstrasser *et al.*, 1991) were incubated for 4 hours with BIO/MOL ubiquapture beads. The beads were washed thoroughly with RIPA buffer and the bead-bound fraction (denoted ‘B’ in the figure) was boiled in loading buffer, analyzed on western blot and compared with the whole cell extract ‘W’ and the un-bound fraction ‘F’. Rad4TAP is visualized using PAP antibody.

(C), as (B), but blot probed with mouse anti-ubiquitin antibody (Zymed laboratories).
blot and probed it with anti-ubiquitin. The results clearly show enrichment of ubiquitylated proteins (Fig. 3C), confirming the functionality of the beads. Whereas the results from the UBA bead experiment imply that Rad4 is ubiquitylated the modification was still present in rad33 cells deleted for any of the genes encoding non-essential E2 ubiquitin conjugases (RAD6, UBC4, UBC5, UBC7, UBC8, UBC11 UBC12, UBC13) (data not shown), indicating that these E2 enzymes are probably not involved in the observed modification.

3.4 Modification of Rad4 is dependent on Rad26.

The notion that Rad4 modification is triggered by UV irradiation suggests a relationship with the NER process. To examine this we checked whether the core NER proteins Rad2 and Rad14 are required for the modification of Rad4. Figure 4A shows that the deletion of neither RAD2 nor RAD14 affects the modification, demonstrating that the incision reaction is not required for the modification event.

In human cells ubiquitylation of XPC is dependent on the UV-DDB complex. UV-DDB functions in complex with Cul4A and Roc1 and acts as a Cullin-RING ubiquitin ligase (Sugasawa et al., 2005). There are no clear sequence homologues of DDB1 or DDB2 in S. cerevisiae but several indications suggest that the Rad7-Rad16 complex may have a similar function. The Rad7-Rad16 complex binds to Cul3 and the elongin C homologue Elc1. This four-protein complex bears the hallmarks of a typical Cullin-

![Western blot analysis](image)

Figure 4

Western blot analysis. TCA extracts of cells containing TAP-tagged Rad4 were analysed on western blot using PAP antibody. Cells were harvested and suspended in PBS, irradiated and resuspended in YPD medium. After 30 minutes TCA extracts of the cells were prepared.

(A) Rad4TAP in rad33 cells, rad33rad2 cells, rad33rad14 cells, rad33rad16 cells and rad33elc1 cells 30 minutes after UV irradiation (+) or mock treatment (-).

(B) Rad4TAP in wildtype cells, rad33 cells or rad33rad26 cells 30 minutes after UV irradiation (+) or mock (-) treatment.
RING ubiquitin ligase and is suggested to be functionally homologous to UV-DDB (Ramsey et al., 2004). Moreover, it was shown that this complex is able to mono-ubiquitylate Rad4 \textit{in vitro}. (Gillette et al., 2006). We studied the possible role of the Rad7-Rad16-Cul3-Elc1 complex in the UV induced modification of Rad4 in \textit{rad33} cells. Figure 4A shows that the modification of Rad4 is not dependent on Rad16 or Elc1 which implies that the modification we observe is regulated in a different way. Surprisingly, the TCR factor Rad26 appeared essential for the increased modification as it was abolished in \textit{rad33} cells deleted for \textit{RAD26} (Fig. 4B). The requirement of Rad26 suggests that Rad4 modification in \textit{rad33} cells depends on the coupling of transcription to NER.

3.5 \textit{Rad33} binds to \textit{Rad4} via \textit{direct interaction}
In large scale interaction screens the Rad33 protein is found to co-precipitate with both the Rad4 and Rad23 proteins (Gavin et al., 2002; Krogan et al., 2006). However, the affinity capture experiments do not reveal whether Rad33 binds Rad4 via a direct interaction or via Rad23 or other proteins. To gain more insight in the binding of Rad33 to the Rad4-Rad23 complex, we assessed interactions between Rad4, Rad23 and Rad33 using a yeast two-hybrid system. Since the full-length Rad4 protein is lethal in \textit{E.coli} (Fleer et al., 1987; Siede and Eckardt-Schupp, 1986), the amino-terminal domain of Rad4 (residues 1-277) and the evolutionary conserved domain between residues 274 and 667 (Bateman et al., 2004; Finn et al., 2006) were tested separately.

Figure 5 shows that there is no interaction of the amino terminal Rad4 fragment (residues 1-277) with either Rad23 or Rad33. Rad4(274-667) binds to both Rad23 and Rad33 (Fig. 5A). On the other hand, Rad33 shows no interaction with Rad23, indicating that Rad33, like Rad23, binds Rad4 directly.

3.6 \textit{Rad33} binds \textit{Rad4} via \textit{conserved residues which are essential for the XPC-Centrin2 interaction.}
No sequence homologue of Rad33 was found in the human genome database. In order to gain insight in the structure of Rad33 we used mGenthreader to predict the structural features of Rad33 and compared the results with a database of solved structures.

\textbf{Figure 5}
\textbf{Two hybrid test.} The \textit{RAD4} region coding for residues 1-277, the \textit{RAD4} region coding for residues 274-667 and the \textit{RAD23} and \textit{RAD33} genes were cloned in the pGBK7 or pGADT7 vectors (Clontech Matchmaker 3) as indicated in the figure. The vectors and constructs were introduced into Y187 and AH109 as described in materials and methods. The cells were assayed for growth on YNB medium selective for the presence of both plasmids (left panel) and on YNB medium selective for transcription activation of the reporter genes (\textit{HIS} and \textit{ADE}) (right panel), which is indicative of interaction of the proteins tested.
The blast search returned several candidates which, albeit with medium or low confidence, showed resemblance with the predicted structure of Rad33. It is noticeable that all proteins retrieved from the database are calmodulin-like proteins. The most similar structure is that of the yeast Cdc31 protein (table 2).

Interestingly, XPC also binds a calmodulin-like protein, Centrin2 (Araki et al., 2001), which is one of the human homologues of Cdc31. The interaction between XPC and Centrin2 has been intensively studied and three amino acids in XPC required for this interaction were identified (Nishi et al., 2005; Popescu et al., 2003; Thompson et al., 2006; Yang et al., 2006). The residues involved in the XPC-Centrin2 interaction are conserved and present in all known Rad4 homologues (Nishi et al., 2005 and Fig. 6A).

The two-hybrid experiments (Fig. 5) show that Rad4 interacts directly with Rad33 and Rad23 via the highly conserved domain between residues 274 and 667 (Bateman et al., 2004; Sonnhammer et al., 1997). In XPC this conserved domain also contains the binding site for both HHR23B and Centrin2. To investigate a possible parallel between the

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<tr>
<td>Myocin light chain</td>
<td>Low</td>
<td>2bl0-C0</td>
<td>Physarum polycephalum</td>
</tr>
<tr>
<td>E-LC</td>
<td>Low</td>
<td>1wdc-C0</td>
<td>Aequipecten irradians</td>
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<tr>
<td>Calcium-binding protein</td>
<td>Low</td>
<td>2scp-A0</td>
<td>Nereis diversicolors</td>
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<tr>
<td>Calcium-binding protein</td>
<td>Low</td>
<td>1jfl-A0</td>
<td>Entamoeba histolytica</td>
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roles of Rad33 and Centrin2 in the yeast and human damage recognition complexes respectively we examined whether the conserved residues in XPC required for binding of Centrin2 to XPC are also implicated in the binding of Rad33 to Rad4.

The three residues W649, L652 and L656 (WLL residues, figure 6A) in the conserved region of Rad4 were replaced by alanines (referred to as the rad4AAA mutation). The RAD4 fragment containing the rad4AAA mutation was cloned in the pGKT7 vector and used for two-hybrid experiments. The Rad4AAA protein does bind Rad23 which implies that the overall conformation has not changed dramatically (Fig. 6B). Interestingly, Rad33 does not interact with the Rad4AAA protein (Fig. 6B) showing that Rad33 binds Rad4 via the WLL motif.

3.7 Cells lacking the Rad4-Rad33 interaction.
To examine the UV sensitivity of cells in which the Rad4-Rad33 interaction is disrupted we introduced the rad4AAA mutation in wildtype cells and in rad33 cells and checked the UV sensitivity of the resulting mutants. Figure 7A shows that the introduction of the rad4AAA mutation leads to moderate UV sensitivity, similar to that of rad33 mutants. Interestingly, the additional deletion of RAD33 in a rad4AAA mutant does not lead to an increase in UV sensitivity (Fig. 7A). This demonstrates that the NER defect of the rad4AAA cells is the specific result of the disrupted Rad4-Rad33 interaction.

The rad4AAA mutation allows us to study the effect of the loss of Rad33 from the Rad4-Rad23 complex on the modification of Rad4, without interfering with any other possible roles of Rad33. The modification of the Rad4AAA protein from UV irradiated wildtype or rad33 cells was compared to the modification of wildtype Rad4. In agreement with earlier experiments (Fig. 2C and D) the high molecular species of Rad4 were observed in UV irradiated rad33 cells but not in wildtype background (Fig. 7B). In lane 6 of figure 7B the modification of the Rad4AAA protein is shown. It is evident that the modification of Rad4AAA is very similar to that of the wildtype Rad4 protein analyzed in rad33 background as seen in lane 4. Importantly, additional deletion of RAD33 in rad4AAA mutants does not alter the modification. This proves that the increase in UV dependent modification of Rad4 in rad33 cells is not caused by an indirect effect linked to the rad33 deletion, but due to the disruption of the interaction between Rad4 and Rad33.

Figure 7.
(A) UV survival droptest. Wildtype, rad4, rad33, rad4AAA and rad4AAArad33 cells were grown for 3 days in YPD. Appropriate dilutions of the cells were spotted on YPD plates and irradiated with UV as indicated. Cells were grown for 3 days in the dark at 30°C. (B) Western blot analysis. TCA extracts of UV irradiated (+) or mock treated (-) wildtype and rad33 cells expressing Rad4TAP or Rad4AAATAP were prepared as described in the materials and methods and were analysed on western blot using PAP antibody. Lanes 1-4 show Rad4TAP in wildtype (lane 1-2) or rad33 (lane 3-4) extracted from UV irradiated (+) or mock (-) treated cells. Lanes 5-8 are similar to lane 1-4, but here cells expressing Rad4AAATAP are analysed.
The Rad4-Rad23 complex is responsible for initial damage recognition in NER in *Saccharomyces cerevisiae* (Guzder *et al.*, 1998; Jansen *et al.*, 1998; Min and Pavletich, 2007). We recently identified Rad33, a NER factor that might also be involved in this process (den Dulk *et al.*, 2006). Rad33 is found in complex with both Rad4 and Rad23 (Krogan *et al.*, 2006). Our data demonstrate that Rad33 binds directly to Rad4 and not to Rad23. Moreover, distinct sites on the Rad4 protein are involved in the binding of Rad33 and Rad23 respectively, indicating that the three proteins might exist in one complex.

We have previously shown that Rad4 steady state levels are lower in cells lacking Rad33 (den Dulk *et al.*, 2006). By analyzing protein levels in the absence of *de novo* protein synthesis we here demonstrate that the decreased amount of Rad4 protein in *rad33* cells are not the result of instability of Rad4. The reduction might be the consequence of lower Rad4 synthesis when Rad33 is absent. In UV irradiated *rad33* cells Rad4 is also stable, thus, in contrast to what is reported on Rad4 in *rad23* cells (Lommel *et al.*, 2002; Ortolan *et al.*, 2004; Xie *et al.*, 2004) our observations show the reduced levels of Rad4 protein in *rad33* cells are relatively stable.

Our studies here show that UV induced modification of Rad4 is strongly increased in *rad33* cells. Since XPC is ubiquitylated upon UV irradiation (Sugasawa *et al.*, 2005; Wang *et al.*, 2005) we examined whether the modification of Rad4 is the result of ubiquitylation as well. We obtained evidence that ubiquitin is involved but we could not unambiguously determine the nature of the modification. None of the non-essential E2 ubiquitin conjugases appear to be involved in the modification and also anti-ubiquitin antibodies from two different suppliers failed to detect the modified Rad4. Also when we precipitated modified Rad4 from cells over-expressing MYC-tagged ubiquitin we yet could not detect any MYC-ubiquitin in the Rad4 precipitates (data not shown). In an alternative approach, beads coated with Ubiquitin Associating (UBA) domains were used. The relative amount of modified Rad4 was increased in the bead-bound fraction, indicating that Rad4 is ubiquitylated. It is noticeable that the higher band of the modification is especially enriched. This band is the faintest of the modified Rad4 in whole cell extracts and hardly visible in Rad4-precipitates. This might explain the difficulty to obtain a signal using anti-ubiquitin (or anti-MYC).

Our observations here do not provide elucidation of the nature of the modification and at this point we cannot exclude that Rad4 is modified by post translational modifications different from ubiquitylation, like sumoylation.

The modification of Rad4 in *rad33* cells is not dependent on the incision reaction since it is not inhibited in the absence of the core NER proteins Rad2 and Rad14. The recently identified Rad7-Rad16-Cul3-Elc1 E3 ligase complex, that is shown to monoubiquitylate Rad4 *in vitro* (Gillette *et al.*, 2006), is also not involved since deletion of *ELC1* or *RAD16* does not affect the modification. Surprisingly, we find that Rad26 is essential for the increased UV induced modification of Rad4, suggesting that the coupling of NER to the transcription machinery is essential for the modification to take place. The faintly visible modification of Rad4 in wildtype cells appears however not dependent on Rad26. Long exposures of the blots showed Rad4 modification in *rad26* cells reminiscent of that in wildtype cells. This type of modification is apparently not
related to Rad26 dependent TCR.

The augmented modification of Rad4 is likely to be related to the NER defect of rad33 cells. However, it is unclear whether this effect is the cause or the consequence of the impaired NER reaction. If the increased modification of Rad4 is assumed to be the (partial) cause of the repair defect in rad33 cells, it is expected that inhibition of the modification event would (partially) suppress the repair defect of rad33 cells. However, this assumption is opposed by the fact that rad33rad26 cells lack Rad4 modification but are severely NER deficient compared to rad33 single mutants. It is therefore more likely that the Rad4 modification is the result rather than the cause of the defective NER in rad33 cells. The modification of Rad4, like ubiquitylation of XPC, might be required for efficient NER of certain lesions. The observation that modified Rad4 is hardly detectable in wildtype cells might indicate that the modified Rad4 is quickly processed in the presence of Rad33. Deletion of RAD33 possibly causes a delay or blockage in the processing of the reaction intermediate involving the modified Rad4, thereby causing a net increase in modified protein.

No clear sequence homologue of Rad33 was identified in higher eukaryotes, however, fold recognition analysis (Jones, 1999) in our study shows structural resemblance of Rad33 to the yeast Cdc31 protein. Cdc31 is the yeast homologue of the human Centrin2 protein. Noticeably, Centrin2 binds to the human Rad4 homologue XPC (Araki et al., 2001). Centrin2 is one of the three identified centrin isoforms in humans which are essential for duplication and segregation of the microtubule organization centers (MTOC), known as the spindle pole bodies (SPB) in yeast (Baum et al., 1986; Middendorp et al., 2000; Salisbury et al., 2002). However, more than 90% of the Centrin proteins in the cell are not associated with the centrosome (Paoletti et al., 1996) indicating involvement of these proteins in other processes.

In recent years a role of Centrin2 in human NER has been described. Addition of Centrin2 to in vitro NER reactions stimulates NER activity, possibly by stabilization of XPC (Araki et al., 2001). The Centrin2-XPC complex is extensively studied and three amino acids in XPC that are essential for the interaction with Centrin2 are identified (Nishi et al., 2005; Popescu et al., 2003). Cells in which the XPC-Centrin2 interaction is disrupted show significantly reduced GGR. Biochemical analysis of XPC, HHR23B and Centrin2 shows that the binding of Centrin2 stimulates the DNA-binding activity of XPC (Bunick et al., 2006). HHR23B has a similar effect but in contrast to Centrin2 is found to dissociate upon the binding of XPC to DNA (Bunick et al., 2006; Nishi et al., 2005; You et al., 2003).

There is yet no indication that Cdc31, the only Centrin homologue in S. cerevisiae, is involved in NER since all the large scale interaction studies performed failed to show an interaction of Cdc31 with Rad4, Rad23 or any other NER protein. In this study we show that Rad33 binds Rad4 via the three amino acids that correspond to the residues required for the XPC-Centrin2 interaction. Importantly, genetic disruption of this interaction results in mutants with a phenotype similar to rad33 deletion cells, showing that the role of Rad33 in NER is completely dependent on its interaction with Rad4. The data presented here and in our previous paper show similarities between the defects of yeast and human cells in which the Rad4-Rad33 and XPC-Centrin2 interaction is disrupted respectively; in both mutants GGR appears to be defective and the protein levels of both Rad4 and XPC are lower compared to wildtype cells. This observation,
combined with the predicted structural resemblance and the fact that Rad33 and Centrin2 bind to Rad4 or XPC via the same conserved motif, might suggest that the role of Rad33 in the Rad4-Rad23 complex is similar to that of Centrin2 in human XPC-HHR23B complex.

A striking resemblance is observed when XPC is modeled on the recently published crystal structure of Rad4 (Min and Pavletich, 2007), suggesting that Rad4 and XPC recognize lesions via a comparable mechanism. The Rad4 protein of which the crystal structure was determined lacks the carboxy-terminal part that is essential for the interaction with Rad33 (Min and Pavletich, 2007). This region is presumed to be unstructured and flexible in both Rad4 and XPC (Charbonnier et al., 2007; Min and Pavletich, 2007). Data derived from the crystal structure of Centrin2 bound to the carboxy-terminal fragment of XPC implies that the binding of Centrin2 stabilizes the unstructured XPC region. Binding of Rad33 to Rad4 might have a comparable effect. The conformational change in the carboxy-terminal part of Rad4 upon binding of Rad33 may stimulate the affinity for damaged DNA, as is shown for XPC by Nishi et al. (Nishi et al., 2005). Since this region is also involved in the XPC-TFIIH interaction (Uchida et al., 2002; Yokoi et al., 2000) it is also suggested that the ability to recruit TFIIH might be altered by binding of Centrin2 (Charbonnier et al., 2007). Given the analogy with Centrin2, Rad33 might be involved in the same mechanism.

The role of Rad23 in the Rad4-Rad23 complex as well as the function of the post-translational modification of Rad4 and XPC is still under debate (Bergink et al., 2007; Reed and Gillette, 2007; Sugasawa, 2006). Yet, several studies imply that Rad23, sumoylation and ubiquitylation cooperate in the tight regulation of the protein levels and the DNA binding properties of Rad4 and XPC, thereby providing flexibility to the NER damage recognition process. Our present data suggest that Rad33 is an additional factor affecting damage recognition by the Rad4-Rad23 complex. The adaptive nature of damage recognition could be essential for quick initiation of NER only when it is required, avoiding interference with other metabolic processes acting on undamaged DNA (Bergink et al., 2007; Bunick et al., 2006; Gillette et al., 2006; Wang et al., 2007). The parallels between Centrin2 and Rad33 might imply that these proteins modulate the function of XPC and Rad4 respectively. The elucidation of their roles will contribute to the understanding of the mechanism of damage recognition and the involvement of post translational modifications in this process.

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References


Rad33 is involved in the modification of Rad4


